Host and environmental risk factors associated with *Cryptosporidium scophthalmi* (Apicomplexa) infection in cultured turbot, *Psetta maxima* (L.) (Pisces, Teleostei)

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Abstract

An epidemiological cohort study of *Cryptosporidium scophthalmi* in cultured turbot *Psettta maxima* L. of Northwestern Spain was conducted along a four-year period. Four different ongrowing cohorts were monitored monthly from introduction into the ongrowing tanks (10-50 g) until reaching market size (400-1400 g). The association of host and environmental factors with five categories of parasite abundance was assessed using a multivariable regression framework. Epidemiological factors assessed here were water temperature, weight, length, month of collection, season, age, origin, condition factor, water filtration, and status to the myxozoan *Enteromyxum scophthalmi* infection. *E. scophthalmi* was included into the analysis because it targets the same organ than *C. scophthalmi* and it was prevalent in the studied population. The multivariable analysis demonstrated the statistically significant association between several factors and parasite abundance. *C. scophthalmi* abundance was associated ($P<0.05$) with age, condition factor, season, and status to *E. scophthalmi* infection. Young animals, with poor condition factor, during spring or summer, and not infected with the myxozoan were more likely to be highly infected by *C. scophthalmi*. Inclusion of these four variables significantly ($P<0.05$) improved the model, compared to the model that did not include any of these epidemiological factors. Increasing levels of *C. scophthalmi* abundance were associated ($P<0.01$) with higher severity of *C. scophthalmi*-compatible lesions. The frequency of distribution of *C. scophthalmi* abundance was clearly right-skewed and fitted a negative binomial distribution, whereas the intensity of infection fitted a Poisson distribution. The quantification of the variance-to-mean ratio stratified by age demonstrated overdispersion for 8-16 months old fish, although this bivariate association is likely affected by several other factors, as suggested by the results of the multivariable analysis. The negative relation between *C. scophthalmi* abundance and status to *E. scophthalmi* infection suggests differences in the transmission, onset, and course of both

infections. The coarse filtration used in some cohorts did not significantly affect the levels of infection. *C. scophthalmi* was probably introduced into the ongrowing tanks mainly with carrier fish, though the involvement of infective oocysts from the water supply cannot be disregarded. Infection prevalence and mean intensity decreased with fish age and a seasonal distribution was found. Results presented here will help to understand the epidemiology of *C. scophthalmi* in turbot, to estimate the expected levels of infection associated to presence or absence of epidemiological factors, and to quantify the impact that the disease may have on susceptible turbot populations. The multivariable model used here is more powerful for exploring associations in cooperative processes than the visual inspection of graphics and can be easily extended to the assessment of epidemiological associations in other population and parasitic diseases.

Key words: epidemiology, multivariable regression model, aquaculture, fish

1. Introduction

Parasites of the genus *Cryptosporidium* are intracellular protozoa that infect epithelial cells of the gastrointestinal and respiratory tracts of a wide variety of vertebrates, including humans (Fayer et al., 1997, 2000a; de Graaf et al., 1999; Dillingham et al., 2002; Tzipori and Ward, 2002; Fayer, 2004; Sunnotel et al., 2006). The characteristics of the host-parasite relationships have been broadly assessed for members of this genus affecting economically important mammalian and avian hosts.

The extensive information available for many members of the genus contrasts with the rather limited knowledge on epidemiological characteristics of the infection caused by *Cryptosporidium* spp. to poikiloterm and particularly piscine species. (Fayer et al., 1997). Two *Cryptosporidium* species, referred to as *C. molnari* and *C. scophthalmi*, have recently been reported from cultured fish. Infection by *C. molnari* was reported in gilthead sea bream *Sparus aurata* L. and Mediterranean sea bass *Dicentrarchus labrax* L. and data on epidemiology and pathological effects are available elsewhere (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla et al., 2005). *C. scophthalmi* was described from turbot, *Psetta maxima* (Alvarez-Pellitero et al., 2004). The different life-cycle stages, from merozoites to oocysts, occur in the intestine, the target organ. Merogonial and gamogonial stages occupy the typical extracytoplasmic position, whereas sporogonial stages are located deep within the epithelium, as in other piscine cryptosporidioses. This turbot coccidian produces a marked histopathological damage that is more evident in severely infected fishes and that can lead to sloughing of epithelial cell remnants and oocysts, or even to detachment of intestinal mucosa.

Epidemiological investigation is an important component in the process of understanding infectious diseases in aquaculture (Georgiadis et al., 2001). Host-parasite

relationships at the population level are complex and many factors related to the microhabitat, i.e., the host, and to the macrohabitat, i.e., the host environment, of the parasite must be considered in order to understand the epidemiological factors associated to the prevalence of and risk for diseases and syndromes (Arneberg et al., 1998; Poulin, 2000). In farmed hosts, culture conditions and management practices are determinants of epidemiological factors, such as host density, that are likely to affect the host-parasite relationship. Multivariable regression is an approach commonly used in analytical epidemiology aimed at measuring the individual and associated effects of multiple factors, forces, and predictors on the levels of disease, while decreasing or eliminating the influence of confounders (Dohoo et al, 2003a) Therefore, epidemiological investigation of disease-affected populations assessed in a multivariable framework provides useful information on the nature and extent at which certain factors or forces may influence the process of infection. This information can ultimately be used to design strategies that are effective in controlling or preventing the introduction of parasitic diseases into susceptible populations.

Turbot, *Psetta maxima*, is one of the main marine fishes farmed in Europe, and its intensive cultivation is hampered by different parasites, including *Cryptosporidium scophthalmi*. Incidental reports and recent routine surveys conducted in turbot cultures of Galicia indicate that *C. scophthalmi* is prevalent in the region (authors' unpublished observations). However, to the best of the authors' knowledge, no epidemiological assessment of *C. scophthalmi* infection in *P. maxima* populations has been published and the importance that this recently described parasite may have for the aquaculture industry is still unknown.

Here, results of an epidemiological assessment of *C. scophthalmi* infection in susceptible populations of turbot from North Western Spain are presented. The study was conducted along a four-year period, from pre-ongrowing to market size and using a cohort study design. The association of host and environmental factors with parasite distribution and

levels of infection was assessed using a multivariable regression framework. Results presented here will contribute to the understanding of the nature and extent at which epidemiological factors are associated with the incidence of *C. scophthalmi* in naturally infected populations of *P. maxima* and will ultimately help to design strategies to control and prevent the spread of the disease.

2. Materials and methods

2.1. Fish

A parasitological cohort study was carried out in the ongrowing facilities of a turbot farm in Galicia, Northwestern Spain. Fish were cultured in a land-based shore site with a pump flow-through seawater supply (37 ‰ salinity), in 600 liter round tanks at approximately 25 kg/m^2 stocking density. Fish were kept in the hatchery until weighting 5 g with water filtered through sequential cartridge filters up to 1 µm and UV-irradiated (25 J/m^2 and 25-30 m^3/h water flow). Fish were then transferred to nursery facilities (with coarse sand-filtered water 50 µm) until weighting about 10 g, when they were introduced into the ongrowing tanks.

2.2. Study design

A cohort study design was used to analyze data and samples collected from June 1997, when the first cohort of turbot was introduced into the on-growing facilities, through December 1999, when the last samples were collected. Data and samples were originally collected, processed, and analyzed to identify factors associated with *Enteromyxum scophthalmi* infection (Quiroga et al., 2006) and were recently re-analyzed with the objectives described here. This type of design is referred to as a retrospective cohort study in classical epidemiology (Dohoo et al, 2003b) and it has the advantage of reducing operative costs while preserving the appropriateness of a prospective cohort design. The total number of studied turbot specimens was 841, divided into six different cohorts (Table 1). Fish from cohorts A, B

and C, from the same broodstock, were hatched at the same farm in which fish were grown (hatchery 1), whereas fish from cohort D came from another hatchery (hatchery 2) and broodstock. To study the influence of water filtration, cohorts C and D were divided into two cohorts, receiving filtered water (CF and DF) and unfiltered water (CUF and DUF), respectively. Water filtration was carried out using a 50 μm-pore sand filter. Temporal changes in intensity and prevalence of infection were studied using the 841 fish of the four cohorts A, B, C, D, whereas statistical analysis were only applied to 620 fish from cohorts C and D for which complete records were available. The sampling scheme is summarized in Table 1.

2.3. Sampling and histological processing

Turbot were sacrificed by chilling on ice and spinal cord severance or by overexposure to the anaesthetic MS-222 (Sigma, St Louis MO, USA), and bled from the caudal vein before the necropsies. Fish were weighed, measured, and necropsied. The digestive tract was excised for fresh and histological examination for parasites. Samples of oesophagus; stomach; anterior, middle and posterior parts of the intestine were fixed in 10% neutral buffered formalin.

For the histological study, fixed tissue samples were embedded in paraffin or Technovit-7100 resin (Kulzer, Heraeus). Paraffin sections (4-5 μm) were routinely stained with hematoxylin-eosin and toluidine-blue and resin sections (1-3 μm) with toluidine blue. Giemsa, Ziehl-Nielsen or PAS staining were occasionally employed to better visualize some stages.

Fish were considered infected when the parasite was detected in histological sections of intestine, which is the target organ of infection (occasional findings in the stomach were registered, but only in fish parasitized in the intestine as well). The number of parasites (ps) present in the microscope fields of at least two sections at 300x magnification was computed

and categorized using a semi-quantitative density scale from 1 to 4, where 1=1-10 ps per field; 2=11-20 ps per field; 3=21-50 ps per field; and 4= >50 ps per field. A value of 0 was used to indicate absence of ps, which denotes that the fish was free from infection. The presence and severity of pathological lesions typical of cryptosporidiosis were also evaluated in the sections and scored from 1 to 3, in which 1 (slight lesions) indicates few parasites, mostly extracytoplasmic, and no evident associated lesions; 2 (moderate lesions) indicates many parasites, some of them intraepithelial without detaching of epithelium; and 3 (severe lesions) indicates many intraepithelial stages with detaching of epithelium and discrete inflammatory reaction.

Throughout this manuscript, the categorized value of ps that included categories 1 to 4 was referred to as *parasite intensity*, whereas the term *parasite abundance* was preferred when categories 0 to 4 were used, i.e., including non-infected fish. The percentage of infected fish in a given cohort and sample was referred to as *prevalence*. Monthly prevalence and mean intensity were calculated and analyzed for all sampled cohorts to evaluate the temporal changes in infection (Table 2). The terms *intensity*, *mean intensity*, *abundance* and *prevalence* were used as in Bush et al. (1997).

2.4. Data analysis

The nature and extent to which selected epidemiological factors were associated with *C. scophthalmi* abundance was assessed in 620 fish from cohorts CF, CUF, DF, and DUF for which complete records were available. Abundance of *C. scophthalmi* (Y) was grouped into five categories (Y = 0, 1, 2, 3, 4), where Y=0 denoted absence of infection and Y=1 to 4 denoted gradually increasing intensities of infection (1 = slight, 4 = severe) (see above). The association between abundance of *C. scophthalmi* infection and severity of *C. scophthalmi*-compatible lesions, which was coded from 0 to 3 (0=no lesions, 1=slight, 2=moderate, 3=severe), was quantified by computing the Jonckheere-Terpstra statistic. This test is a non-

parametric extension of an ANOVA test, suitable for cases in which the categories of the variable used to categorize the response, in this case, abundance, has been ordered in an increasing order of magnitude, here, from 0 to 4.

A multivariable regression model for ordinal data (McCullagh, 1980) was used to quantify the association between *C. scophthalmi* abundance and epidemiological factors hypothesized to influence the presence of the parasite. The approach was aimed at identifying the set of epidemiological factors that best described the *C. scophthalmi* infection, as measured by the cumulative probability associated with the five categories of abundance (Y = 0, 1, 2, 3, 4). Because the distribution of Y was right-skewed, in other words, with high probability posed in the category denoting absence of infection, cumulative probabilities (C) were transformed using a function of the form $-\log[-\log(C)]$.

The 10 epidemiological factors assessed here were water temperature (ºC), weight (g), length (cm), month of collection (where a value of one designated the month of inception of the project and the largest value designated the last month when samples were collected), season (summer, fall, winter, spring), age (months), origin (same hatchery and broodstock, other hatchery and broodstock), condition factor (measured as the ratio between weight and the third power of the length), the intestinal myxozoan *Enteromyxum scophthalmi* infection (yes, no), and water filtration (yes, no). *E. scophthalmi* infection was included in the analysis due to its enteric location and pathological concern. Epidemiological factors significantly associated ($P<0.05$) with *C. scophthalmi* infection were retained in the final model. Intensity of the association was quantified by computing the slope of the association (beta). Values of beta > 0 indicated positive association (risk factors) and values of beta < 0 indicated negative association (protective factors), whereas values of beta = 0 indicated absence of association.

Significance of the contribution of the epidemiological factors to the model was assessed by computing a Chi-square test to compare the -2 log-likelihood values for the model

that did not include any epidemiological factor with the value estimated for the model that included the epidemiological factors significantly ($P<0.05$) associated with the disease. A value of $P<0.05$ was considered evidence that the inclusion of the selected variables improved the model (McCullagh and Nelder, 1989). Goodness-of-fit of the model was assessed by computing Pearson's chi-square test to compare the values observed with those predicted by the final model. A large P-value ($P>0.05$) was considered evidence of good model fitness. Nagelkerke's coefficient of determination (R^2) was computed to estimate the proportion of the variation of Y that was associated with the epidemiological factors, with large R^2 values, up to a maximum of 1, indicating that most of the variation was explained by the model (Nagelkerke, 1991). All tests were performed using SPSS version 15.0.1, SPSS Inc., Chicago IL, 2006.

In order to study the dispersion pattern and the age-abundance profile of *C. scophthalmi*, the variance-to-mean ratio (VMR) was calculated for the total sampled fish and stratified by age. In addition, the frequency distribution of the parasite within the sampled hosts was computed.

3. **Results**

3.1. Temporal changes in infection

The temporal progression of infection was followed in the four studied cohorts (Table 2). Fish from cohort A, introduced in the ongrowing tanks in June 97, were not found parasitized at the first sampling in Autumn 97 (December), and showed low infection prevalence in Winter 98 (January to March). Conversely, fish from cohort B, introduced in October 97, showed high prevalence and mean intensity of infection from the first sampling in Autumn 97 (November) and alos in Winter 98, whereas the parasite was not found in Spring 98. Fish from cohorts C and D, both introduced into the on-growing tanks in April 98 showed high

prevalences (81-97.7 %) and mean intensities in Spring 98, just after introduction. Prevalence and mean intensity decreased progressively in further samplings until Winter 99, whereas in Spring 99 a moderate rise occurred in CF, DF and DUF fish. Prevalence and mean intensity were higher in D than in C fish. No clear differences were detected between fish receiving filtered or unfiltered water in C, whereas the parasite was more prevalent in DUF fish (Table 2).

3.2. Dispersion pattern

Two hundred and forty one (prevalence = 38.9 %) of the 620 fishes included in the analysis were infected with *C. scophthalmi*. Parasite abundance approximated a negative binomial distribution with parameters $r = 1$ and $p = 0.5641$, i.e., with 25% of the samples having an intensity of infection larger or equal to 1 (Fig. 1). A Poisson distribution with $\mu=1.98$ best described the pattern of parasite intensity.

3.3. Epidemiological factors associated with C. scophthalmi abundance

Abundance of *C. scophthalmi* infection was positively associated (Jonckheere-Terpstra statistic = 16.524; $P<0.01$) with severity of *C. scophthalmi*-compatible lesions (Fig. 2). Abundance of *C. scophthalmi* infection was associated ($P<0.05$) with age, condition factor, season, and status to *Enteromyxum scophthalmi* infection, with the most abundant infections observed in fish that were young, in poor condition, not infected by *E. scophthalmi*, and in spring and summer (Table 3).

Inclusion of these four variables significantly ($P<0.05$) improved the model, compared to the model that did not include any epidemiological factor. The model fitted the data adequately (Pearson's Chi-square = 2208, $P = 0.820$). Almost half of the variation in the dependent variable was explained by the variables included in the model ($R^2 = 0.46$). The bivariate relation between the variables that fitted the final models and abundance of *C. scophthalmi* infection has been graphed in Figs. 3-6. Fig. 7 depicts the age- and season-related

variation in *C. scophthalmi* prevalence, which helps to visualize the combined effect of two of the epidemiological factors in the dynamics of the disease. Prevalence decreases siginificantly with age and it was highest in spring and summer ($P<0.05$, Table 3). Therefore, prevalence in >15 month old (m. o.) fish sampled in spring or summer was, in average, higher than the prevalence in 10-15 m.o. fish sampled in fall or winter, but lower than the prevalence observed in 4-9 m. o. fish sampled the previous spring and summer (Fig. 7).

The value of VMR varied with age. Values of VMR>1, indicating overdispersion were obtained for 8-16 m. o. fish, whereas younger and older fish presented VMR < 1 (Fig. 8).

DISCUSSION

The influence of host and culture conditions on the epidemiology of *Cryptosporidium scophthalmi* infection in turbot was assessed. The use of a multivariable analysis allowed for the quantification of the extent at which certain risk factors were associated with *C. scophthalmi* abundance and helped to establish their potential influence in cryptosporidiosis onset and progress. This is the first time that the association between *C. scophthalmi* abundance and factors hypothesized to influence the course of the disease is quantified in a multivariable framework. Quantification of the strength of associations is important because it leads to a better understanding of the relative importance that different factors and forces have on the epidemiology of the disease.

Fish from cohorts B, C, and D were found highly infected from the first sampling, shortly after introduction into the ongrowing system, which suggests that they were already infected at the time of entrance. Furthermore, fish have been observed to be already infected at hatchery and nursery facilities, being prevalence as high as 60-100 % in 3 m. o. (7-8 g) fish (Alvarez-Pellitero et al., 1999, and authors unpublished results). Thus, transmission of *C.*

289 *scophthalmi* probably takes place through the water supply, as reported for *C. molnari* and
290 other cryptosporidia infections (Sitjà-Bobadilla et al., 2005; Brookes et al., 2004; Fayer,
291 2004). Filtration and UV irradiation of water in the hatchery and nursery did not prevent the
292 introduction of infective oocysts into the system. A similar treatment of water supply in the
293 hatchery did not prevent the infection by *Cryptosporidium molnari* in gilthead sea bream,
294 *Sparus aurata* (Sitjà-Bobadilla et al., 2005). UV irradiation (ranging between 20 and 120
295 mJ/cm^2) and conventional filtration of tap water for human consumption have shown to be
296 unreliable for the removal of *Cryptosporidium* spp. (Fayer et al. 2000a; Betancourt and Rose,
297 2004). In addition to the water supply, the involvement of live food in the transmission of *C.*
298 *scophthalmi* in the larval stages should not be ruled out. Rotifers can ingest *C. parvum*
299 oocysts (Fayer et al., 2000b). Moreover, *Artemia*, which is a common live food used in
300 marine fish larval development, is capable of spreading *Cryptosporidum* oocysts and its
301 involvement in the transmission of cryptosporidiosis in cultured fish has been suggested
302 (Méndez-Hermida et al., 2006, 2007).

303 In the population analyzed here, *C. scophthalmi* seems to have been introduced into
304 the ongrowing tanks by carrier fish, as suggested by the positive disease status observed in
305 individuals sampled at the time of introduction. However, the involvement of infective
306 oocysts present in the water supply in ongrowing facilities cannot be disregarded, as
307 suggested by the prevalence increase observed in May in CF, DF and DUF fish. The coarse
308 filtration used in cohorts CF and DF did not significantly affect the levels of infection.
309 Transmission and dispersal of fish cryptosporidia are facilitated by the aquatic habitat and the
310 frequent releasing of fully sporulated oocysts with mucus casts or faeces. Although it has not
311 been demonstrated, horizontal transmission for *C. scophthalmi* is likely to occur, as in the
312 case of the piscine *C. molnari* (Sitjà-Bobadilla and Alvarez-Pellitero, 2003). In addition,
313 different aquatic organisms, such as filter feeding shellfish, could act as reservoirs for *C.*

314 *scophthalmi*, as it has been reported for other *Crypstoporidium* spp. (reviewed in King and
315 Monis, 2006; Fayer, 2004).

316 Analysis of our results shows a significant decrease in *C. scophthalmi* abundance with
317 fish age (beta = -0.17, Table 3). This result is in accordance with the observation that the
318 disease was less prevalent in fish from cohort A, which was examined from 10 to 17 months
319 of age, compared to fish from cohorts B, C and D, that were studied starting at 5 months of
320 age (Table 2). In cohorts C and D, prevalence reached 100 % in the first sampling, just after
321 introduction. In addition, a progressive decrease in infection prevalence and mean intensity
322 was detected in all cohorts along the study period. These results confirmed the age
323 distribution of *Cryptosporidium* spp. reported in other hosts. The piscine *C. molnari* showed
324 the highest prevalence levels in preongrowing and early ongrowing fish, with a trend to
325 decrease with fish weight (Sitjà-Bobadilla et al., 2005), and a similar trend occurs in other
326 piscine cryptosporidioses (reviewed in Sitjà-Bobadilla et al., 2005). Non-piscine
327 cryptosporidiosis show similar age distribution, as infections are usually high in neonates and
328 young, and less prevalent in adults, both in mammals and in poultry species (Atwill et al.,
329 1999; de Graaf et al, 1999; Guselle et al., 2003; Thompson et al., 2005). In contrast with this
330 negative association of cryptosporidioses with age, the most commonly observed pattern for
331 metazoan parasites is the increase in prevalence and intensity with age or size of the host
332 (Zelmer and Arai, 1998; Poulin, 2000; Thomas, 2002). A non-linear infection pattern, with a
333 peak at a particular age and size of the host and a subsequent decrease in older animals, has
334 also been described for some fish protozoans and myxoporeans (Rintamäki et al., 1997;
335 Palenzuela et al., 1999; Gbankoto et al., 2003), including the turbot parasite *Enteromyxum*
336 *scophthalmi* (Quiroga et al., 2006). Such pattern was also found in the piscine *C. molnari*, and
337 it probably occurs in *C. scophthalmi*, as deduced from the high infection prevalence and
338 intensity found in larval fish (Alvarez-Pellitero et al., 1999 and authors'unpublished results).

In piscine cryptosporidioses, age-dependent differential exposure could be involved, since the concentration of infective stages due to the use of recirculating systems or live food at the hatchery could lead to a higher parasite load in these growing steps. This notwithstanding, the decrease of infection prevalence and intensity in older individuals could be related to decreased susceptibility due to acquired immunity, as it has been suggested in mammalian and human cryptosporidioses (Current, 1989).

The frequency distribution of *C. scophthalmi* was right-skewed and fitted a negative binomial distribution. Over-dispersion is the model described for the great majority of parasites (Shaw et al., 1998). Here, the distribution of *C. scophthalmi* infection was over-dispersed for fish of intermediate age, as demonstrated by the quantification of the VMR stratified by age. It must be considered, however, that the influence of several factors in the distribution of infection abundance, such as condition factor, season, and infection with *Enteromyxum scophthalmi*, may have affected the bivariate association between age and VMR (Fig. 8). Duerr et al. (2003) suggested that interpretation of age-intensity profiles derived from cooperating processes is, at least, difficult, due to the influence that other epidemiological factors may impose on the distribution of infection. In the study here we have overcome this problem by formulating a multivariable framework, which allowed for the quantification of the association between factors and infection while adjusting by the presence or absence of other factors significantly associated with the disease. Therefore, the approach used here is more effective, informative, and powerful for exploring associations in cooperative processes than the visual inspection of the graphics alone. Moreover, the adjusted multivariable model may be used to predict the expected levels of *C. scophthalmi* abundance in infected populations for given values of the epidemiological factors. This information may be used, for example, to obtain estimates of the expected impact of the disease in populations

known to be infected and for which information on the epidemiological factors is known, but for which it is not feasible to perform long term longitudinal studies as the one presented here.

A seasonal influence on the levels of *C. scophthalmi* infection was estimated here, with maximum levels of prevalence, abundance, and intensity in spring and summer (Tables 2 and 3). The piscine *C. molnari* also exhibited maximum levels of infection in spring (Sitjà-Bobadilla et al., 2005). Seasonality occurs in natural infections by other fish coccidians, which are generally more prevalent in spring (reviewed in Steinhagen and Davies, 2008). Seasonal patterns have also been reported for non-piscine cryptosporidioses, being generally predominant in warm seasons (de Graaf et al., 1999; Sturdee et al., 2003). Temperature is probably one of the most important factors involved in cryptosporidia seasonality, although other factors, such as infective status of fish at the introduction in the ongrowing tanks, availability of infective stages, host density, or a combination of different processes may also contribute to the observed seasonal pattern.

Increasing levels of *C. scophthalmi* infections were significantly associated with high severity of *Cryptosporidium* lesions. In addition, abundance of infection was low in fish with high condition factor. Such results point to a pathological significance of this infection, though the effect in a decreased body condition as a contributor to possible mortalities, remains to be demonstrated.

The negative association between the infection intensities of *C. scophthalmi* and *E. scophthalmi* is probably due to differences in the transmission, onset and course of both infections rather than to a true competition between both parasites. *C. scophthalmi* seemed to be introduced into the ongrowing system mostly through carrier fish, and the infection progressively decreased with fish age/weight. In contrast, fish are free from *E. scophthalmi* infection when introduced in the ongrowing system, and the first infections are usually detected at least three months after introduction. Culture conditions favour the transmission

and dispersion of this myxozoan, and thus infection levels progressively increase in growing fish (Quiroga et al., 2006), with an opposite pattern to that observed for *C. scophthalmi*.

In conclusion, *C. scophthalmi* infection was confirmed in turbot cohorts from their introduction into the ongrowing systems and abundance of infection in the fish was negatively associated with age, concurrent infection with *E. scophthalmi,* and condition factor and positively associated with spring and summer. The results presented here will help to understand the epidemiology of *C. scophthalmi* in turbot, to produce estimates of expected levels of infection in infected populations based on the presence or absence of risk factors, and to quantify the impact that the disease may have on susceptible turbot populations of Galicia.

Acknowledgements

Funding for this study was provided by the EU and the Spanish Ministerio de Ciencia y Tecnología through research grants FEDER 1FD97-0679 and AGL2001-2241. Additional support was obtained from Stolt Sea Farm S. A. We are thankful to J. Monfort from IATS, and to M. C. Carreira Valle from the School of Veterinary Medicine of Lugo (University of Santiago) for assistance in processing histological samples.

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514

515 **Figure legends**

516 Fig. 1. *Cryptosporidium scophthalmi* abundance coded into five categories (0 = nil, 1
517 = slight, 4 = severe). Black lines indicate the proportion of fishes observed for a given
518 category of density. Grey bars depict the expected probability distribution using a negative
519 binomial distribution fitted with parameters $r = 1$ and $p=0.564$

520 Fig. 2. Association between abundance and severity of lesions due to *Cryptosporidium*
521 *scophthalmi* infection. Abundance and severity of lesions have been coded, respectively, into
522 five (0 = nil, 1 = slight, 4 = severe) and four (0 = no lesions, 3 = severe lesions) categories.
523 The black square and the upper and lower extremes of the black lines indicate, respectively,
524 the median value, first quartile and fourth quartile of the severity of lesions observed for a
525 given category of infection.

526 Fig. 3. Association between condition factor and *Cryptosporidium scophthalmi* abundance.
527 Abundance has been coded into five categories (0 = nil, 1 = slight, 4 = severe). The black
528 square and the upper and lower extremes of the black lines indicate, respectively, the mean
529 value, first quartile and fourth quartile of the condition factor observed for a given category of
530 abundance.

531 Fig. 4. Association between age (in months) and *Cryptosporidium scophthalmi* abundance.
532 See figure 3 for reference.

533 Fig. 5. Association between season and *Cryptosporidium scophthalmi* abundance. See figure
534 3 for reference.

535 Fig. 6. Proportion of *Enteromyxum scophthalmi*-infected fishes (dark bar) stratified by
536 *Cryptosporidium scophtalmi* abundance (0 = nil, 1 = slight, 4 = severe).

537 Fig. 7. Prevalence of *Cryptosporidum scophthalmi* infection in fish from cohorts C and D
538 stratified by age and season.

539 Fig. 8. Age-related variation of the variance-to-mean ratio (VMR) of the *Cryptosporidium*
540 *scophthalmi* abundance. Raw values (solid line) and three-month moving average (point line)
541 are shown.
542

543 Table 1: Sampling conditions of turbot (*Psetta maxima*) cohorts assessed for *Cryptosporidium*
544 *scophthalmi* infection.

Cohort	Date of introduction	Origin[1]	No. sampled fish	First sampling	Sampling period (months)	Water filtration
A	Jun 1997	H1	61	Dec 1997	6	No
B	Oct 1997	H1	148	Nov 1997	8	No
CF	Apr 1998	H1	96	Apr 1998	14	Yes
CUF	Apr 1998	H1	228	Apr 1998	11	No
DF	Apr 1998	H2	100	Apr 1998	14	Yes
DUF	Apr 1998	H2	208	Apr 1998	20	No

545 [1]H1: Hatchery of the farm were the study was conducted; H2: A hatchery from a different
546 farm. Cohorts A, B and C: the same broodstock and H1; cohort D: another broodstock and
547 H2. CF and DF: in filtered water; CUF and DUF: in unfiltered water.

548 Table 2. Details on sampling schedule, temperature (T), number of sampled fish (n), prevalence (P) and mean intensity (MI) of *Cryptosporidium*

549 *scophthalmi* infection in fish from cohorts A, B, C and D. CF and DF: in filtered water; CUF and DUF: in unfiltered water. Empty cells indicate

550 no available data

Sample date	T ºC	Fish cohort																	
		A			B			C						D					
								CF			CUF			DF			DUF		
		n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI
Nov 97	16.5				15	86.7	2.8												
Dec 97	14.7	11	0	0	32	62.5	2.2												
Jan 98	13.5	20	10	2	24	79.2	1.6												
Feb 98	14.3	16	31.3	2.2	15	20	3												
Mar 98	15.6	10	30	2	21	66.7	1.5												
Apr 98	14.0				21	0	0	4	100	3.8	14	100	2.6	4	100	3.3	14	100	3.6
May 98	14.3	4	0	0	16	0	0				11	100	2.1	4	100	2.5	14	100	2.4
Jun 98	13.8				4	0	0	12	75	2.2	28	67.8	2.2	8	87.5	2.7	16	93.7	1.9
Jul 98	14.0							4	75	1	31	54.8	1.5	4	0	0	18	72.2	1.6
Aug 98	14.5							4	75	1.3	18	38.9	1.6	4	50	1.5	14	92.8	2.1

Sep 98	16.5	8	37.5	1.3	40	15	1.5	8	87.5	1.1	28	46.4	1.3
Oct 98	15.3	12	8.3	1	25	4	2	12	41.7	1	8	0	0
Nov 98	15.0	4	25	1	15	0	0	4	0	0	4	0	0
Dec 98	10.7	12	0	0	12	0	0	12	8.3	1	12	50	1
Jan 99	11.4	8	12.5	1	18	0	0	8	0	0	8	12.5	1
Feb 99	12.8	8	0	0	16	6.3	2	8	0	0	8	12.5	2
Mar 99	12.6	4	25	1				4	0	0	8	12.5	1
Apr 99	13.0	8	25	1				8	0	0	8	25	1
May 99	15.2	8	62.5	1				12	33.3	1	8	50	1
Jun 99	15.1										4	0	0
Jul 99	14.9										8	25	2
Aug 99	16.3										4	50	2
Sept 99	16.1										8	0	0
Nov 99	16.5										8	0	0
Dec 99	12.0										8	0	0

551

552 Table 2. Details on sampling schedule, temperature (T), number of sampled fish (n), prevalence (P) and mean intensity (MI) of *Cryptosporidium*

553 *scophthalmi* infection in fish from cohorts A, B, C and D. CF and DF: in filtered water; CUF and DUF: in unfiltered water. Empty cells indicate

554 no available data

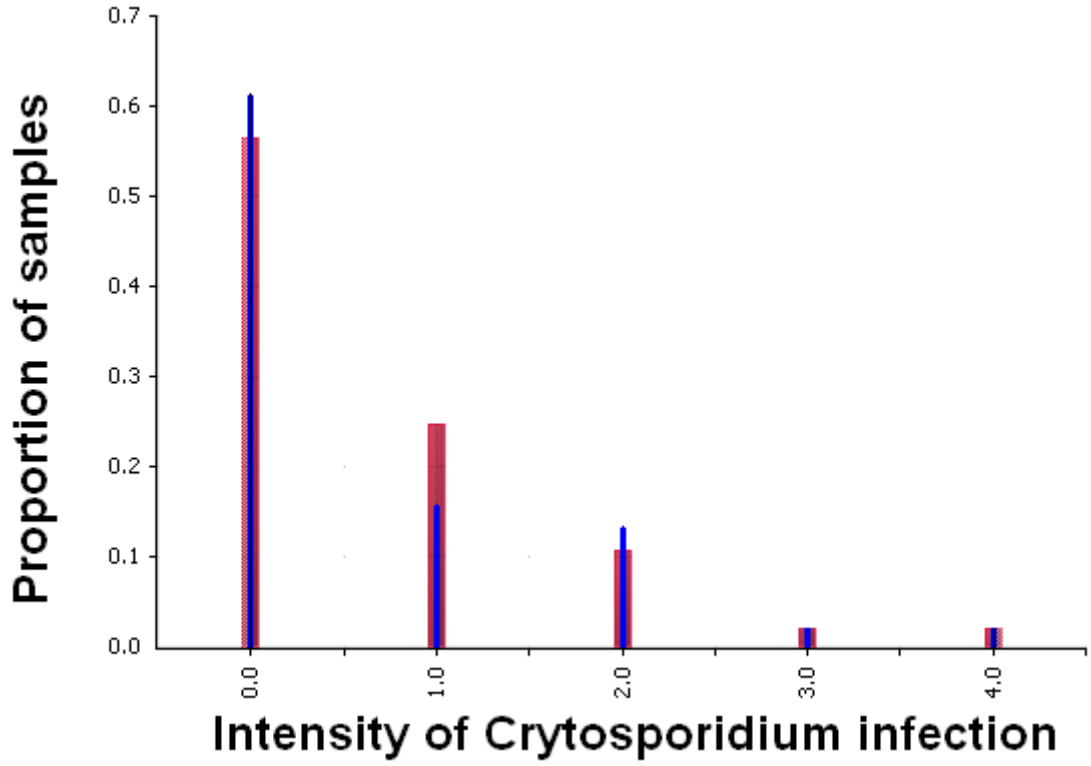
Sample date	T ºC	Fish cohort																
		A			B			C						D				
								CF			CUF			DF			DUF	
		n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI
Autumn 97	16.5- 14.7	1	0	0	4	70.	2.											
Winter 98	13.5-15.6	4	21.	2.	6	60	1.											
Spring 98	14.0-13.8	4	0	0	4	0	0	1	81.3	2.	5	83	2.	1	93.	2.	4	97.	2.
Summer 98	14.0-16.5							1	56.2	1.	8	33.	1.	1	56.	1.	6	65	1.
Autumn 98	15.3-10.7							2	7.1	1	5	1.9	2	2	21.	1	2	25	1
Winter 99	11.4-12.6							2	10	1	3	2.9	2	2	0	0	2	12.	1.
Spring 99	13.0-15.1							1	43.8	1				1	20	1	2	30	1
Summer 99	14.9-16.1																2	20	2
Autumn 99	16.5-12																1	0	0

555

Table 3. Association between *C. scophthalmi* abundance in turbot and selected risk factors quantified in a cohort study in NW Spain. P-values < 0.05 indicate a significant association.

Variable	Slope (beta)	P-value	95% Confidence Interval of the slope	
Age	-0.17	<0.001	-0.22	-0.12
Condition factor	-12.65	0.001	-19.94	-5.36
Spring	1.74	<0.001	0.77	2.72
Summer	1.02	0.036	0.07	1.96
Fall	0.33	0.539	-0.71	1.36
Absence of *E. scophthalmi*	2.22	<0.001	1.05	3.39

Fig. 1


Proportion of samples
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
1.0
2.0
3.0
4.0
Intensity of Crytosporidium infection

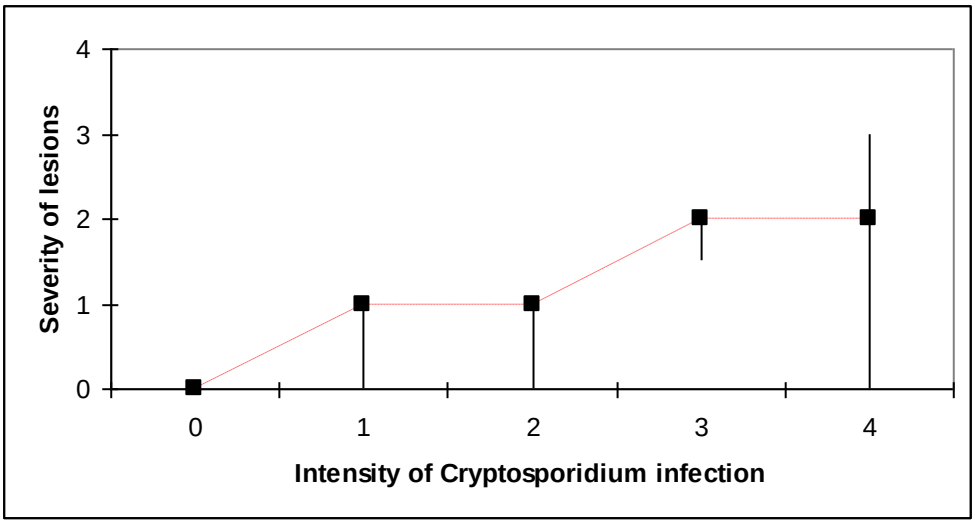

Severity of lesions
4
3
2
1
0
0
1
2
3
4
Intensity of Cryptosporidium infection

Fig. 2

Fig. 3

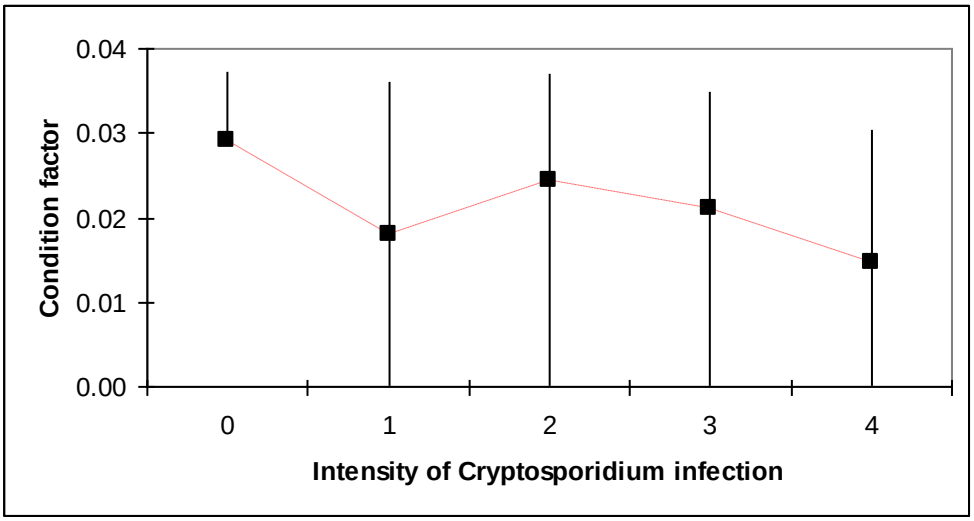

0.04
0.03
0.02
0.01
0.00
Condition factor
0
1
2
3
4
Intensity of Cryptosporidium infection

Fig. 4

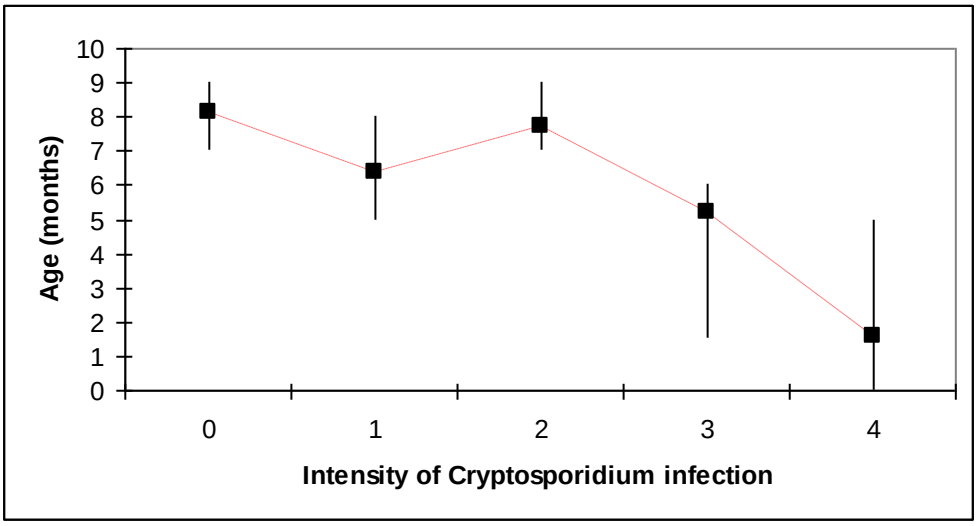

Age (months)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
Intensity of Cryptosporidium infection

Fig. 5

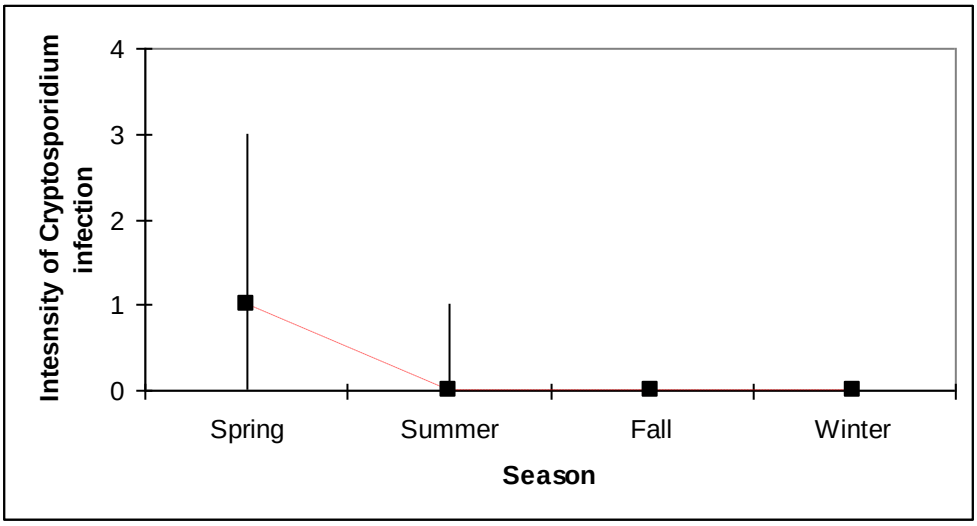

Intesnsity of Cryptosporidium infection
4
3
2
1
0
Spring
Summer
Fall
Winter
Season

Fig. 6

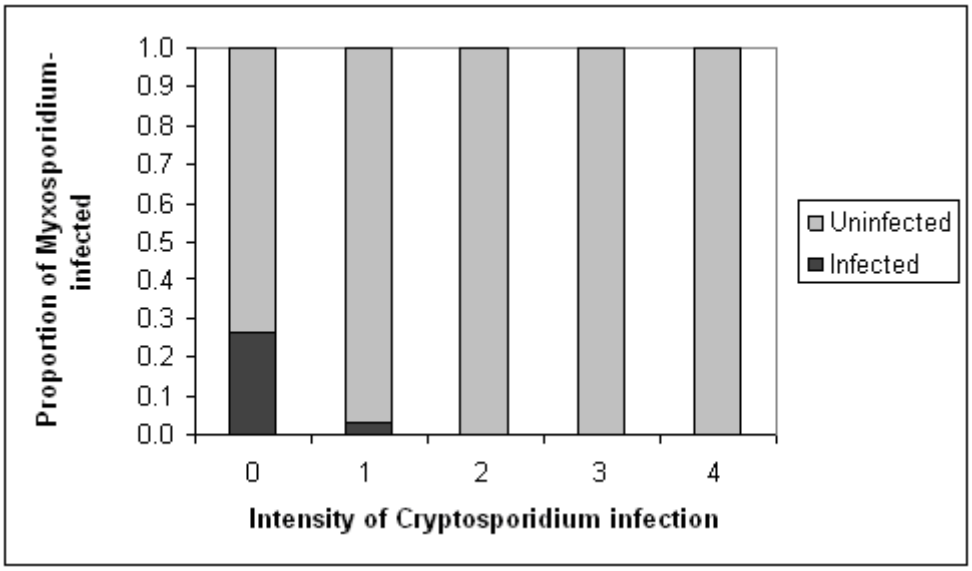

Proportion of Myxosporidium-infected
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
Intensity of Cryptosporidium infection
Uninfected
Infected

Fig 7

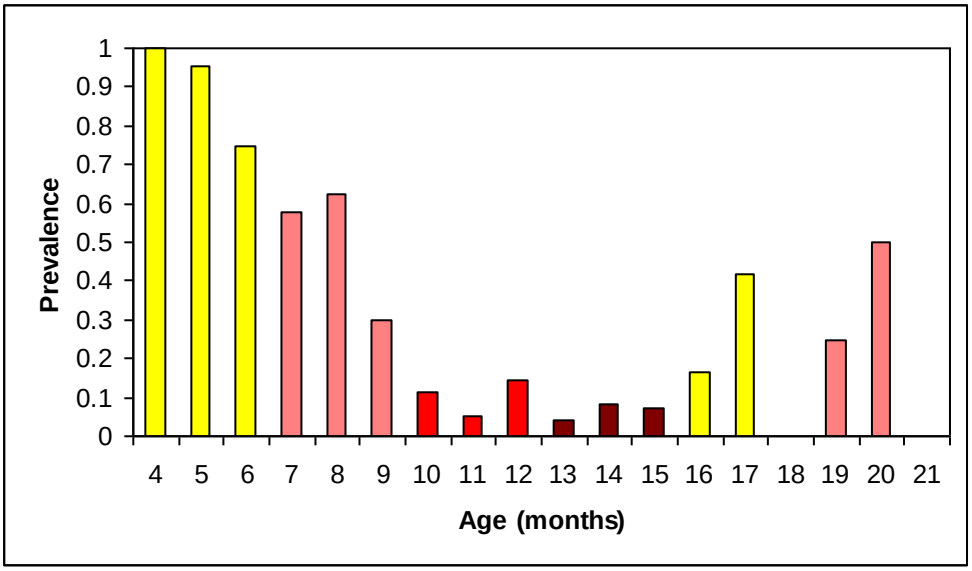
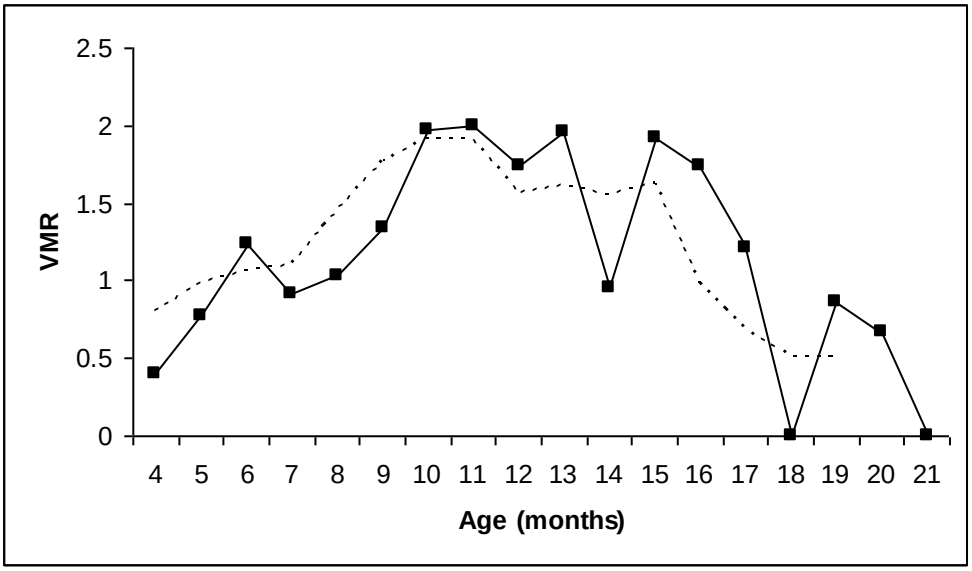

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Age (months)

Fig 8


2.5
2
1.5
1
0.5
0
VMR
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Age (months)